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A STUDY OF *IN VITRO* OVULATION IN THE EASTERN SPADEFoot TOAD, *SCAPHIOPUS H. HOLBROOKI*¹

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The first experiments concerned with the induction of ovulation by implantation of pituitary glands were performed on mammals (Ascheim, 1926; Smith, 1926; Zondek, 1926). Since that date many workers have demonstrated that pituitary materials will induce ovulation in representatives from most of the vertebrate classes. Rugh (1935), showed that excised ovaries from *Rana pipiens* would ovulate in Ringer's solution when their donors were injected with pituitary materials some hours before the removal of the ovary. The first experiments exclusively employing *in vitro* ovulation were conducted by Heilbrunn *et al.* (1939), in which whole untreated ovaries from *R. pipiens* were suspended in Ringer's solution containing macerated pituitaries. Ovulation ensued and all the eggs which ovulated showed first polar-body formation. Ryan and Grant (1940) helped to establish the normalcy of *in vitro* ovulation by artificially fertilizing eggs which had been ovulated by this technique and placed in the coelomic cavity of an ovariectomized female, where they passed through the oviducts receiving a jelly coat. Such eggs cleaved normally and most hatched into swimming tadpoles. In 1945, Wright described some of the quantitative and biochemical aspects of *in vitro* ovulation in *R. pipiens*. Wright and Hisaw (1946) showed that mammalian pituitary extracts evoked ovulation, both *in vitro* and *in vivo* in the leopard frog. Wright (1946) reported that hypophysectomy

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brought about an increased responsiveness of the ovary to *in vitro* ovulation, this probably being caused by the release of gonadotropins in the operation. This hypothesis was strengthened by the increase in ovulation *in vitro* following the immersion of ovarian pieces in serum taken from hypophysectomized frogs (Wright and Macintyre, 1950). Rondell (1953) used the *in vitro* ovulation technique to study the metabolic systems operative in ovulation in *R. pipiens* but was unable to show the precise method of operation.

Although the writer's primary interest has been a general study of the physiological and ecological factors involved in the breeding response of the eastern spadefoot, the present discussion will be confined to a number of factors affecting ovulation *in vitro*.

MATERIALS AND METHODS

Over 1000 spadefoot toads, *Scaphiopus h. holbrooki*, collected from a single area near Gainesville, Florida, were used in this study. In general, the *in vitro* technique used was similar to that described by Wright (1945). Ovarian donors weighed from 10 to 20 grams and had head widths varying from 18 to 23 mm. The ovaries were removed and cut into small pieces containing 20 to 35 eggs each. These fragments were suspended by cotton threads in stoppered vials containing 10 ml. of Holtfreter's solution (Fig. 1). Ovulation was induced by the addition of spadefoot pituitary homogenate.

The extripation of the pituitary involved severing the cranium from the body, removing the oral mucosa, inserting fine-pointed scissors lateral to the medulla and cutting out the ventral portion of the brain case. The exposed pituitary lies just posterior to the optic chiasma. The round glandular anterior lobe is easily removed from the intermediate and posterior lobes with fine-pointed forceps. Pituitaries were macerated with a small mortar and pestle and diluted with an appropriate amount of Holtfreter's solution.

Ovulation was never observed in blank controls of Holtfreter's solution used in each experiment to check for possible spontaneous ovulation. All tests were allowed to stand for 18 hours and were conducted at a room temperature of approximately 23°C., unless otherwise indicated.

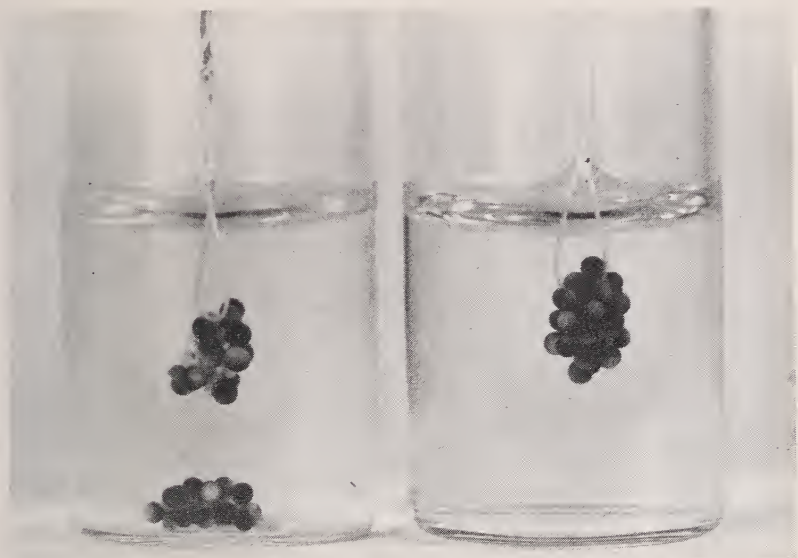


Figure 1. Control vial (right) with ovarian fragment suspended in Holtfreter's solution and experimental vial (left) containing pituitary homogenate showing fallen eggs which have been ovulated.

Percentages of ovulation were determined by dividing the number of eggs ovulated (*i.e.*, falling to the bottom of the vial) by the total number of eggs present in the ovarian fragment, and multiplying by 100.

RESULTS

Nine factors affecting *in vitro* ovulation in the eastern spadefoot were considered.

1. *Comparative Potency of Male and Female Pituitary.*—Since approximately 50% of the individuals collected were males which could be utilized as an additional pituitary source, a test was designed to compare the relative potency of male and female glands. Pituitaries from five male toads proved to be 81% as potent as that from five equal-sized females. Equivalent weights of dried pituitary showed the male 76% as effective as the female in eliciting ovulation.

2. *Individual Variation.*—Both Wright (1945) and Rondell (1953) using *R. pipiens* found considerable variation in ovulation

percentages. To test the variation between individuals, eight pieces of ovary from six equal-sized females were exposed to a standardized pituitary solution. A statistical comparison between ovulation means showed no significant differences. In subsequent experiments, statistical comparisons showing significant differences were considered valid.

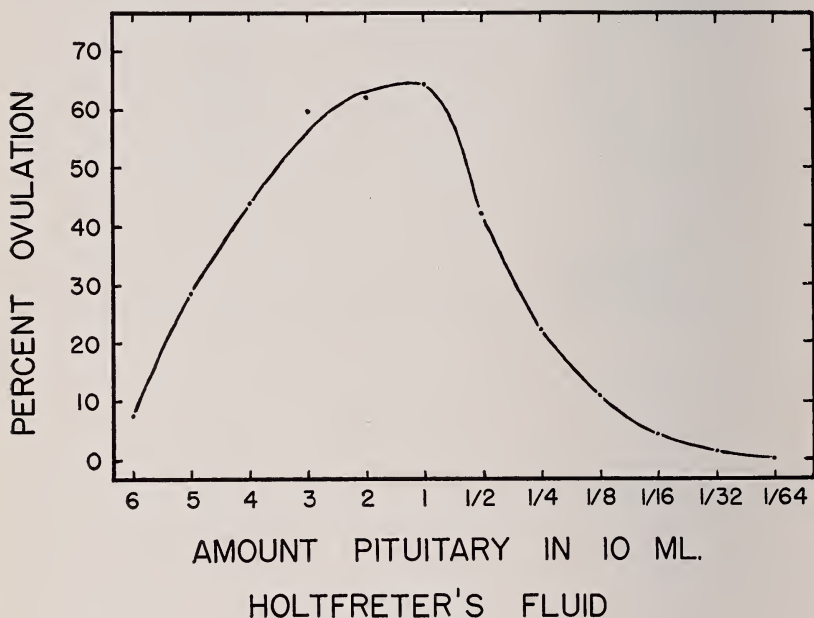


Figure 2. *In vitro* ovulation results obtained from varied concentrations of pituitary suspensions. Each point on the graph represents the average percentage of ovulation obtained in six vials.

3. *Relation of Toad Size.*—Eggs from a single individual were distributed among a 42 vial series, employing pituitaries from males ranging in head width from 17 to 23 mm. The mean percentage of ovulation for each size class was as follows: 17mm.—29%; 18 mm.—37%; 19 mm.—36%; 20 mm.—29%; 21 mm.—32%; 22 mm.—37%; 23 mm.—36%. The correlation between the size of pituitary donors and percentage of ovulation was low ($r = +0.11$) and lacked statistical significance due to the small sample. Utilizing the ovulation data from all experiments in which females were not subjected to experimental conditions (350 individual tests),

a low negative correlation ($r = -0.16$) was found; this test proved to be highly significant ($P < 0.01$). From these data it appears that the size of the pituitary donor has little influence upon the inducement of ovulation.

4. *Effect of Pituitary Dilution.*—In order to establish the optimal amount of pituitary material needed to produce maximal ovulation, a series of pituitary dilutions ranging from 6 glands to 1/64 of a gland in 10 ml. of Holtfreter's solution was effected. The ovulation percentages (Fig. 2) for the various pituitary dilutions were as follows: 6 glands—7.5%; 5—28.5%; 4—44.4%; 3—59.5%; 2—61.8%; 1—64.5%; 1/2—42.1%; 1/4—22.5%; 1/8—11.1%; 1/16—4.3%; 1/32—1.4%; and 1/64—0.0%. Ovulation was found to be inhibited by both high and low concentrations of pituitary homogenate and maximal results occurred with one pituitary in 10 ml. of fluid. These results are in accord with Wright (1945) who found inhibition of ovulation with *R. pipiens* when using high concentrations of pituitary.

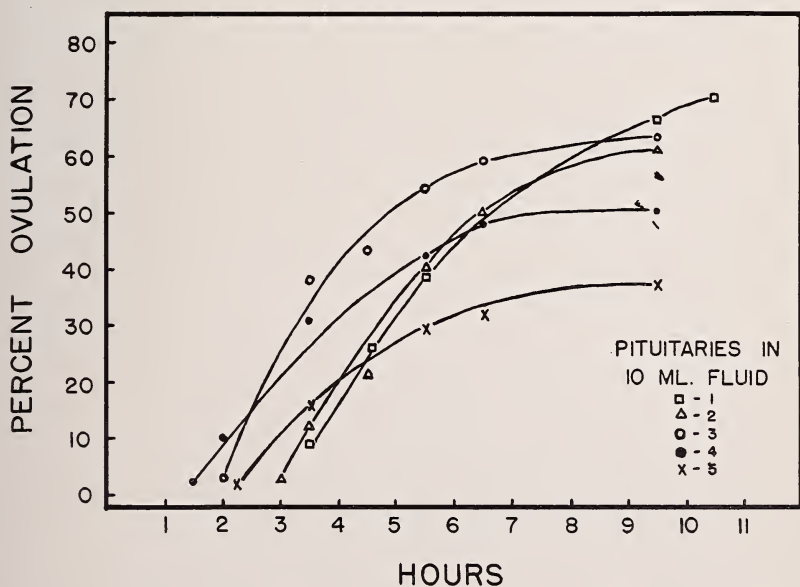


Figure 3. Effect of pituitary dilution upon the time required to initiate ovulation *in vitro*. Lower dilutions effected higher percentages of ovulation.

5. *Time Relationships*.—To determine the time required for the ovulation process to reach an equilibrium, experimentation was conducted in which the hourly progress of ovulation was recorded. Ovulation began about 3½ hours after the experiment was initiated, with approximately 75% of the eggs being ovulated in the following 3 hours, and was completed in 11½ hours. Maximal ovulation occurred in the sixth hour. Wright (1945) found that lower dilutions of pituitary took longer to initiate ovulation in the leopard frog. To test this for the spadefoot, a series of pituitary concentrations varying from 1 to 5 glands was effected. Ovulation was evoked in the 4 gland homogenate in 1½ hours; 3 gland—2 hours; 5—2½ hours; 2—3 hours; and 1—3½ hours (Fig. 3).

6. *Effect of Light*.—Since experiments were to be conducted at all hours, it seemed important to determine any effect of light upon the process. Ovaries removed under a photographic safe light and held in complete darkness showed no significant differ-

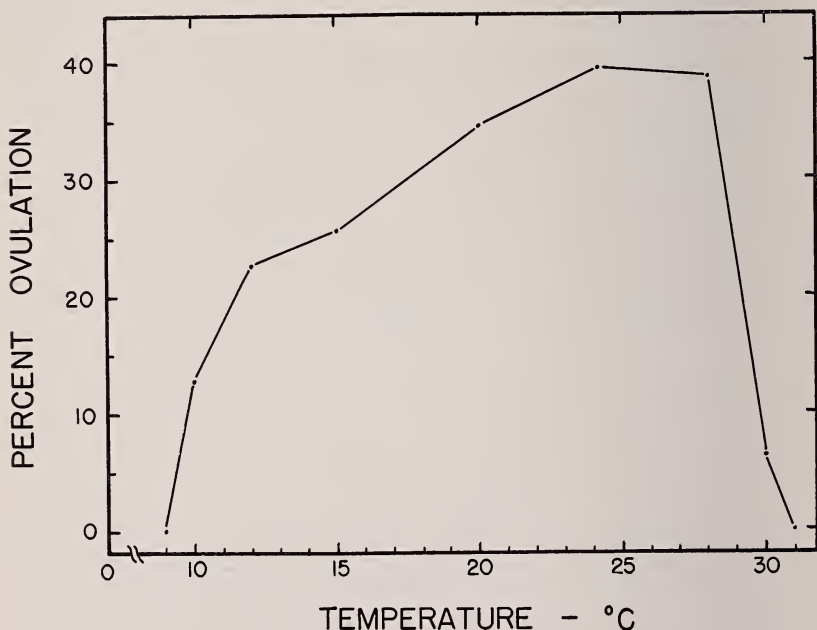


Figure 4. *In vitro* ovulation after exposure of ovarian fragments to a range of temperatures. Note the sudden inhibitory effect at higher temperatures.

ences in ovulation percentages from those exposed to constant light.

7. *Effect of Temperature.*—Wright (1945) found that *in vitro* ovulation in *R. pipiens* took place between temperatures of 17 to 32°C., with an optimum at about 22°C., and that no ovulation occurred at 12° or 37°C. A series of experiments on the spadefoot established definite temperature limits, showing that ovulation occurred between 10° and 30°C., with complete inhibition at 9° and 31°C. (Fig. 4).

8. *Effect of pH.*—Since little is known of the influence of pH upon the biocatalytic action of hormones, it seemed important to determine the effect of variable pH concentrations on the *in vitro* ovulation process. A phosphate buffering system (0.07M) was prepared in which the ratio of monobasic to dibasic sodium phosphate was varied to produce a pH gradient from 5.9 to 8.9. Each solution was secondarily checked with the Beckman pH meter after the addition of the pituitary homogenate. Ovulation *in vitro* occurred over a relatively wide range of hydrogen ion concentrations with maximal activity at 7.3. Complete inhibition was found below 6.5 and above 8.5 (Fig. 5). Rondell (1953), using *R. pipiens*, found that *in vitro* ovulation took place between pH values of 6.6 and 8.2, with complete inhibition at 6.0.

9. *Effect of Heteroplastic Pituitary Materials.*—Creaser and Gorbman (1939) after thoroughly reviewing the literature on induced ovulation in Amphibia, pointed out that the effectiveness of a gonadotropic hormone from a foreign species tends to vary directly with the phylogenetic affinity of the donor and recipient species. With this generalization in view, experimentation was conducted to establish the effects of heteroplastic pituitary materials upon the *in vitro* process.

Since the size and hormone titer of the pituitaries varied considerably, it was impossible to utilize the ovulation results for quantitative comparisons. The results were therefore listed simply as being positive or negative. The *in vitro* ovulation mechanism was found quite sensitive to heteroplastic pituitary homogenates. Ovulation was evoked using pituitary materials from the following: Amphibia (*Rana clamitans*, *R. pipiens* *sphenocephala*, *Microhyla c. carolinensis*, *Bufo t. terrestris*, *Hyla gratiosa*, *H. c. cinerea*, *Desmognathus fuscus auriculatus*); Reptilia (*Natrix taxispilota*,

Sceloporus u. undulatus); Aves (*Gallus gallus*); Mammalia (*Sus scrofa*, *Ovis aries*). Negative results were obtained only in the Osteichthys (*Cynoscion nebulosus*, *Sciaenops ocellatus*) and with human chorionic gonadotropin extracts from pregnancy urine.

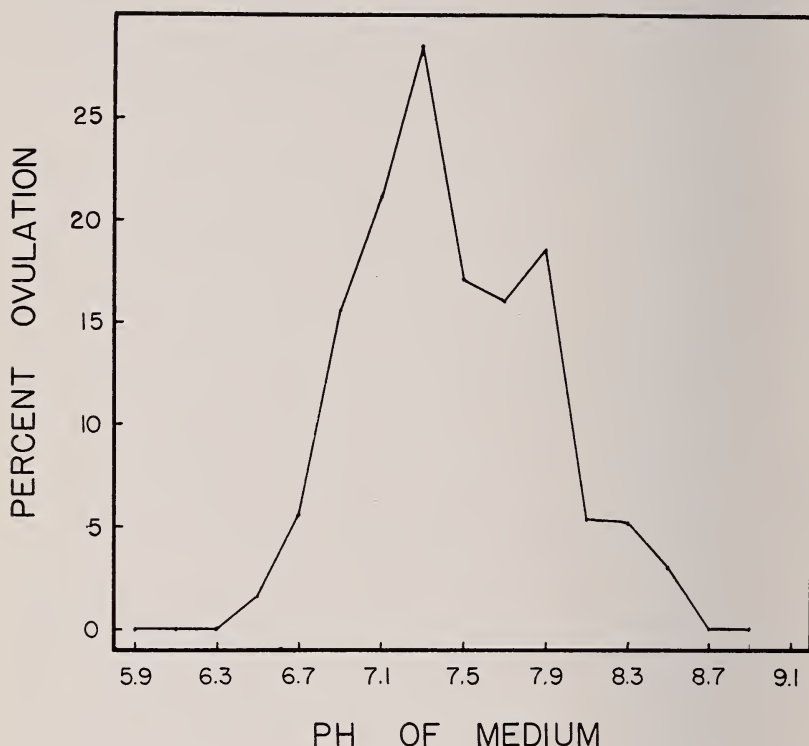


Figure 5. Effect of hydrogen ion concentrations upon *in vitro* ovulation.

DISCUSSION

The question might be raised as to the validity of the *in vitro* ovulation process. Heilbrunn *et al.* (1939) pointed out that eggs ovulating *in vitro* showed polar body formation as normally ovulated ova. In 1940 Ryan and Grant successfully fertilized eggs ovulated *in vitro* after they had received jelly membranes from passing through the oviducts of an ovariectomized female. Wright (1945) showed that the relationship between percentages of ovulation and pituitary concentration is the same for both *in vivo* and

in vitro techniques. The inhibition of *in vitro* ovulation due to large concentrations of pituitary, as shown by Wright (1945) and this work, is in accord with the observations of Foster *et al.* (1937) on the rabbit. Further, there is a striking similarity between the optimum (74°F.) and the upper (86°) and lower (50°) limiting temperatures in the *in vitro* tests and the range of temperatures at which Pearson (1955) found the spadefoot active; optimum, 69°; maximum, 84°, and minimum, 50°F. Also, in close accord with *in vitro* temperature ranges, are temperature records from 34 breeding choruses where spadefoots bred at temperatures ranging from 47° to 83°, with a mean of 71°F. Finally, it is well known that ovulation *in vivo* is stimulated by gonadotropic factors and that ovulation *in vitro* occurs only where pituitary has been used. Since the parallelism between *in vitro* and *in vivo* ovulation is so striking, it seems safe to conclude that the *in vitro* technique affords a valid method for studying ovulation in anurans.

In the spadefoot, the male pituitary was found to be about 78% as potent as the female gland, whereas Rugh (1948) reported that male *R. pipiens* pituitary had only about half the potency of the female pituitary. The difference between these two potency values may be solely specific. However, if this is not the case the present work would seem to be quantitatively more accurate since Rugh's work tested the intact females and was more or less concerned with an all or none response.

Whereas Wright (1945) obtained maximal ovulation in the leopard frog using 1/8 pituitary in 10 ml. of fluid, 1 gland in 10 ml. was required for the spadefoot. In both studies the number of eggs used was approximately the same. Also, the vitellus is almost identical in size for *R. pipiens* (1.3-2.0 mm.) and *S. h. holbrooki* (1.4-2.0 mm.). The difference is probably attributable to the greater size of the *pars distalis* of the leopard frog since the anterior lobe of the latter is two or three times that of the spadefoot. The larger gland would be expected to yield a higher hormone titer than the smaller spadefoot pituitary. The comparatively crude comparisons of gland size merely point up the need for standardized methods of hormone titer determination.

The shorter time required to initiate the onset of ovulation *in vitro* in the spadefoot (3½ hours) as compared to the leopard frog (10 hours) may possibly be due to an over-all acceleration

of reproductive metabolism in the spadefoot. Trowbridge (1941) reported that the rate of embryonic and larval development is more rapid for the spadefoot than that reported in any other amphibian, and that the cell divisions are among the most rapid ever recorded.

Although Wright and Hisaw (1946) employed sheep pituitary extract for their *in vitro* work with *R. pipiens*, the present work is the first to make a wide utilization of heteroplastic pituitary materials. The negative results obtained with piscine pituitary are in accord with the majority of studies in which fish pituitaries were employed for the induction of *in vivo* ovulation in Amphibia (Creaser and Gorbman, 1935, 1939; Rostand, 1934). The low percentage of ovulation using salamander pituitary is in agreement with the work of Adams and Granger (1938) who found that *in vivo* ovulation in *R. pipiens* could only be induced with large numbers of *Triturus* pituitaries. The use of snake and lizard pituitary is the first successful attempt at stimulating gonad activity in Amphibia with reptilian glands. Although Stein (1934) and Witschi *et al.* (1937) induced ovulation in the newt *Triturus* using bird pituitary, the present study is the first to successfully utilize avian pituitary to induce ovulation in the Anura. The failure of chorionic gonadotropin from human pregnancy urine to initiate ovulation *in vitro* in *Scaphiopus* is not surprising, since Creaser and Gorbman (1939) point out that 9 of 12 anuran species failed to ovulate when subjected to human pregnancy urine. It is interesting to note that it is impossible to induce spermiation in the male spadefoot by the injection of human pregnancy urine (Hansen, 1951) or commercial human chorionic gonadotropin (Knepton, 1951).

SUMMARY

1. A study of *in vitro* ovulation employing 1007 male and female eastern spadefoots (*Scaphiopus h. holbrooki*) was conducted from May 1954 through July 1955.
2. Ovarian fragments were suspended on cotton threads in stoppered vials containing pituitary materials macerated in Holtfreter's solution. Percentages of ovulation were determined by dividing the number of eggs ovulated by the total number of eggs present in the ovarian fragment and multiplying by 100.

3. Male pituitary was found to be approximately 78% as potent as female pituitary in evoking ovulation *in vitro*.
4. Although individual variation in ovulation percentages were relatively high, no significant differences were obtained.
5. In experiments involving 371 *in vitro* tests, a very low correlation was found between the size of the pituitary donor and the degree of ovulation induced.
6. No relationship was found between the size of ovarian donors and the percentage of ovulation.
7. A dilution of 1 pituitary in 10 ml. of fluid evoked maximal *in vitro* ovulation, while concentrations above and below this dilution produced lower percentages of ovulation.
8. Using a dilution of 1 gland in 10 ml. of solution, ovulation began in 3½ hours and was completed after 11½ hours. Ovulation was most rapid in the 3 hours following its initiation, with a maximal peak during the third hour.
9. Light and darkness had no apparent effect upon the *in vitro* process.
10. *In vitro* ovulation occurred between 10° and 30°C., with an optimum temperature at about 24°C. Complete inhibition occurred at 9° and 31°C.
11. Ovulation *in vitro* occurred between pH values of 6.5 and 8.5, with maximal activity at 7.3, in a 0.07M sodium phosphate buffering system.
12. Heteroplastic pituitary materials from amphibian, reptilian, avian, and mammalian donors evoked ovulations, while piscine pituitary and human chorionic gonadotropin reacted negatively. The present study was the first to successfully utilize reptilian and avian pituitary for the induction of ovulation in the Anura.

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